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Carmen Contreras-Sesvold

Abstract

Title of Thesis: Reactive Astrocytes: Phenotypic and Functional Characteristics and Astrocytes as Neural Stem Cells

Carmen Contreras-Sesvold, Master of Science, 2006

Thesis directed by Dr. Aviva Symes, Department of Neuroscience

Injury to the CNS elicits a rapid response from the injured environment. The most significant response is that from astrocytes which undergo an activation process and become reactive astrocytes. This causes phenotypic as well as functional changes maintain the tissue integrity, protect adjacent areas, and enable repair processes. However, this process does impede axonal regeneration. Additionally, astrocytes clearly demonstrate the self-renewing multipotential. The function of glial dedifferentiation lends itself to increased study. Emerging data suggests glial cells must dedifferentiate to an astrocyte-like morphology before becoming capable of neural generation.

REACTIVE ASTROCYTES: PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS AND ASTROCYTES AS NEURAL STEM CELLS

By

Carmen Contreras-Sesvold

Thesis submitted to the Faculty of the Department of Neuroscience

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Table of Contents

Approval Sheet i

Copyright Statement ii

Abstract iii

Title Page iv

Table of Contents v

Table of Figures vii

Reactive Astrocytes 1

- Supportive Roles of Astrocytes in CNS 1
- Astrocytes and Brain Injury 3

Phenotypical Hallmarks of Reactive Astrocytes 4

- Reactive Gliosis and Expression of Intermediate Filaments 4
- Hypertrophy and Reactive Astrocytes 8
- Extracellular Matrix Proteins 9

Functional Hallmarks of Reactive Astrocytes 13

- Inflammation 13
- Excitotoxicity 16
- Edema 18
- Blood Brain Barrier 23
- Glial Scarring 23
- Heterogeneity of Reactive Astrocyte Response 26
- Reactive Astrocyte Detrimental and Beneficial Roles Summary 28

Astrocytes as Neural Stem Cells in the CNS 30

Introduction Astrocytes as Adult Neural Stem Cells 30

Criteria for Stem Cell Identification 31

Brain Marrow 32

Advances in the Identification of Neural Stem Cells 34

Astrocytes can Control Neural Induction of Stem Cells 36

The Origins of Neural Stem Cells 38

Subependyma and Neural Stem Cell Origin 38

Subventricular Zone Astrocytes are Neural Stem Cells 39

Subgranular Layer Astrocytes are Neural Stem Cells 41

Glial Cell Dedifferentiation and Neural Stem Cell Progenitors 45

Astrocytes as Adult Neural Stem Cells 47

Conclusion 48

Citations 50

Table of Figures

Neural Stem Cell Type and Description 35

Sequence of Stem Cell Proliferation 40

Reactive Astrocytes

Supportive Roles of Astrocytes in CNS

Many supportive roles and physiological processes of the nervous system are attributed to astrocytes. These contributions include roles in neuronal development and differentiation, maintenance of the blood-brain barrier (BBB), synapse modification and maintenance, and in neurotransmission.

In early development, radial glia provide the temporal and spatial pathway for neurons to migrate to their respective locations (Beasley et al., 2002). In addition to axon guidance (Hatten, 1990; Frangou et al., 2002), and increasing axonal diameter (Sanchez et al., 1996), glia provide trophic support (Hatten and Mason, 1990; Sivron et al., 1993) and modulate axonal pruning (Broadie, 2004).

Evidence supports astrocyte induction, maintenance, and modulation of the BBB (Kettenmann and Ransom, 1995). In the mature organism, astroglial processes, which rarely extend beyond a 50-µm radius, maintain contact with blood vessels, neurons, and other glial cell types, allowing them to exert largely local control. Studies demonstrate astrocytes are able to induce BBB properties in non-neural endothelial cells *in vivo* (Janzer and Raff, 1987). They can also modulate the BBB structure by inducing formation of and enhancing the structure of tight intercellular junctions between capillary endothelial cells and by regulating the expression and function of several endothelial transporters and specialized enzyme systems (Dehouck et al., 1990; Rubin et al., 1991; Sun and O'Donnell, 1996; Hayashi et al., 1997; Schinkel, 1999; Sobue et al., 1999; McAllister et al., 2001; Haseloff et al., 2005).

Growing evidence supports astrocyte involvement in regulation of synaptic strength and plasticity, and that they provide a mechanism for synaptic cross-talk (Pascual et al., 2005). Astrocyte release of ATP has been shown to enhance the dynamic range for long-term potentiation and mediated activity-dependent heterosynaptic depression (Perea and Araque, 2006). Initial studies reveal astrocytes can affect the activity of distant neurons integrated in different neuronal circuits. This is accomplished through their networks of Ca^{2+} wave propagation (Garcia-Segura and McCarthy, 2004) and by the release of signaling molecules such as ATP. It has also been recently demonstrated *in vitro* that cultured astrocytes can release glutamate within exocytosis vesicles via Ca^{2+} dependent mechanisms (Anlauf and Derouiche, 2005). Emerging evidence supports astrocytes as cellular elements involved in information processing by the nervous system (Perea and Araque, 2006) This evidence is based on the existence of bi-directional communication between astrocytes and neurons, in which the astrocyte Ca^{2+} signals mediate signal transduction (Araque et al., 1999; Vesce et al., 1999; Araque et al., 2001; Auld and Robitaille, 2003; Newman, 2003).

Astrocytes can maintain the tissue integrity following central nervous system (CNS) damage and play an important role in recovery response. While the ultimate loss of CNS function is normally attributed to neuronal loss or dysfunction, dysfunction of glia and its ability to maintain homeostasis has been shown to contribute to neuronal damage (Seifert et al., 2006). Reports show that glia function can also mitigate or ameliorate clinical outcome following CNS insult

(Seifert et al., 2006). Astrocytes activated by damage demonstrate intricate region specific differences in function which reflect region specific cellular interactions between neurons and glial cell populations (Ridet et al., 1997). However, there is an incomplete understanding of the protective role of the activated astrocytes in the contribution to neurological pathology.

Astrocytes and Brain Injury

Cerebral vascular accident (CVA) and traumatic brain injury (TBI) elicit a rapid response from the injured microenvironment. The most significant response is that from astrocytes. In this arena, astrocytes undergo an activation process and become reactive to the changes in the microenvironment. This activation, which is in an endogenous process of the CNS to respond to brain insult (Streit et al., 2004) causes phenotypic as well as functional changes in these “reactive astrocytes”. Phenotypically, the changes documented in reactive astrocytes can be characterized as an increased expression if intermediate filaments, hypertrophy, and increased production of extracellular membrane components. Functional hallmarks can be best described as mediation of inflammation, excitotoxicity, edema, blood brain barrier, and formation of the glial scar. Thus, the activation of astrocytes plays a significant role in the repair of the CNS in response to injury.

Phenotypical Hallmarks of Reactive Astrocytes

Reactive Gliosis and Expression of Intermediate Filaments

Following CNS injury cytoplasmic enlargement of astrocytes becomes prominent with the generation of new, thicker, and longer cytoplasmic processes (Panickar and Norenberg, 2005). Accumulation of 2 glial intermediate filaments (IF) glial fibrillary acidic protein (GFAP) and Vimentin (VIM) becomes prominent at this time (Nathaniel and Nathaniel, 1981; Petito et al., 1990; Schmidt-Kastner et al., 1990; Panickar and Norenberg, 2005). This pronounced up-regulation of IFs has lead several investigators to create transgenic animal models to dissect the role that GFAP and VIM play in reactive astrocyte response. One such model includes transgenic mice that carry full copies of the human GFAP gene driven by its own promoter. This mouse line constitutively over-expressed GFAP and was used to evaluate whether over-expression was sufficient to induce astrocyte hypertrophy in the absence of CNS damage. Mice with the highest GFAP expression die by the second postnatal week (Eng et al., 1998; Messing et al., 1998). Moreover, astrocytes of these mice went beyond simple hypertrophy and formed complex intracytoplasmic aggregates of intermediate filaments and small stress proteins. They were also described as hypertrophic, with up-regulation of small heat-shock proteins, and contained inclusion bodies identical both histologically and antigenically to the Rosenthal fibers of Alexander's disease (Messing et al., 1998).

GFAP-null mice were engineered by 4 independent groups to further dissect the role of GFAP in gliosis (Gomi et al., 1995; Pekny et al., 1995; Liedtke et al.,

1996; McCall et al., 1996). These mice displayed normal development and reproduction and with few exceptions also demonstrated normal CNS tissue organization and blood-brain barrier. Furthermore, these mice had normal life span, activity, motion, and memory (Pekny et al., 1995). In response to a variety of CNS traumas, these mice demonstrate responses similar to wild type animals, revealing that up-regulation of GFAP expression is not necessary for the reactive gliosis response (Pekny et al., 1995). These results suggest that other IF can compensate for the lack of GFAP (Pekny et al., 1995; Wu and Schwartz, 1998). Ultrastructural studies revealed that VIM was up-regulated in these transgenic mice and that together with nestin created IFs in the absence of GFAP immunoreactivity (Eliasson et al., 1999).

In contrast, the loss of GFAP in these mice reveals a high susceptibility to cerebral ischemia when initiated by intraarterial suture occlusion of the middle cerebral artery (MCAo) (Nawashiro et al., 2000). Data revealed that 48 hours after combined permanent MCAo and 15 minutes following transient carotid artery occlusion (CAo) $GFAP^{-/-}$ mice had a significantly larger cortical infarct volume as compared to wild-type controls. Laser-Doppler flowmetry revealed that the $GFAP^{-/-}$ mice had a more extensive and profound decrease in cortical cerebral blood flow within 2 minutes after MCAo and CAo. Thus, suggesting an important role GFAP in the progression of ischemic brain damage after focal cerebral ischemia with partial reperfusion (Nawashiro et al., 2000).

The contribution of VIM, another IF protein which is up-regulated in the development of gliosis, was investigated by an *in vitro* study in which infection

with retroviruses containing constructs for sense and antisense cDNA for rat VIM was performed (Lin and Cai, 2004). It showed that antisense cDNA for VIM could both markedly decrease the expression of VIM and also inhibit the expression of GFAP in reactive astrocytes (Lin and Cai, 2004). Suggesting the presence of VIM is necessary for the expression modulation of GFAP and development of gliosis.

VIM null mice were engineered to determine the contribution of this IF to the reactive astrocyte response (Colucci-Guyon et al., 1994). Animals homozygous for this mutation developed and reproduced without an obvious phenotype. Moreover, the absence of compensatory expression of other intermediate filament proteins was also reported. Studies of cultured $VIM^{-/-}$ astrocytes showed that the GFAP network is disrupted (Galou et al., 1996). However, transfection of these astrocytes with a VIM cDNA restored the normal assembly of the GFAP network (Galou et al., 1996). It was further demonstrated i*n vivo* that in the absence of VIM, GFAP can form IF bundles but the structure is aberrant (Eliasson et al., 1999). GFAP expression after stab wound-induced astrogliosis was further examined in these mice. In support of earlier *in vitro* reports, examination in this study revealed that GFAP can assemble aberrant IFs in the absence of VIM (Galou et al., 1996; Eliasson et al., 1999). However, the reactive astrocyte response was reportedly similar to that of wild type once more suggesting the existence of a compensatory mechanism (Pekny et al., 1999).

To evaluate the contribution of both GFAP and VIM to reactive gliosis a $GFAP^{-/-}$ $VIM^{-/-}$ was created (Eliasson et al., 1999). Comparing the IF structures of mouse model, and the $GFAP^{-/-}$ and $VIM^{-/-}$ mice found protein structure

partnerships. It was reported that VIM can partner with GFAP and/or nestin to create IFs. Under certain circumstances GFAP can create IFs alone (Eliasson et al., 1999; Ribotta et al., 2004). However, IFs cannot be formed by VIM-VIM, nestin-nestin, or nestin-GFAP protein assemblies (Eliasson et al., 1999). $GFAP^{-/-}$ $VIM^{-/-}$ mice in response to injury cannot form IFs even when nestin is expressed and are thus devoid of IFs in their reactive state (Pekny et al., 1995).

In summary, GFAP expression in not necessary for the reactive gliosis response but its loss of expression increases the susceptibility to cerebral ischemia (Nawashiro et al., 2000). The loss of VIM, can inhibit the expression of GFAP in reactive astrocytes (Lin and Cai, 2004) and is associated with a abnormal structure of GFAP assembly (Galou et al., 1996 ; Eliasson et al., 1999). Loss of both GFAP and VIM results in the inability to form IFs during reactive gliosis (Pekny et al., 1995). This work further demonstrated that there is a compensatory mechanism in IF formation and identified possible protein partnerships. These partnerships were VIM can partner with GFAP or nestin and in certain cases GFAP can create assemblies alone, but in this investigation, no other partnerships were possible.

Hypertrophy and Reactive Astrocytes

Immediately after CVA or TBI, surviving astrocytes adjacent to the injured tissue undergo a process of hypertrophy and proliferation (Norenberg et al., 2005). The phenotypic hallmarks of this process are described as an increase in the number of mitochondria and volume of the rough endoplasmic reticulum, and enlargement of the Golgi complex in keeping with evidence of increased protein synthesis (Hori et al., 1994). The nuclei of these astrocytes also become enlarged and pale (Jayakumar et al., 2006). Moreover, there is an increase in nuclear size and irregular nuclear outlines along with dispersion of chromatin. These morphologic findings are in character with metabolically active cells (Ridet et al., 1997; Panickar and Norenberg, 2005).

Work examining reactive astrocyte hypertrophy in response to CVA and TBI confirms this description. Immunohistochemical analysis of murine hemispheres 3 days after MCAo showed within the ischemic penumbra activated astrocytes were enlarged with elongated and laminated cytoplasmic processes (Kajihara et al., 2001). With increasing time, these changes became more prominent. At 7 days cells showed marked hypertrophy with highly GFAP immunoreactive and expanded perivascular end-feet (Kajihara et al., 2001). The $GFAP^{-/-}$ $VIM^{-/-}$ mouse model displayed less characteristic results. TBI experiments using this model in concert with an entorhinal cortex lesion found that these reactive astrocytes showed only a limited hypertrophy of cell processes. Instead, many processes were shorter and not straight, although the volume of neuropil reached by a single astrocyte was the same as in wild-type mice (Wilhelmsson et al., 2004).

Extracellular Matrix Proteins

The up-regulation of extracellular matrix molecules (ECM) has been investigated in reactive gliosis. Three of the most studied ECMs are chondroitin sulfate proteoglycans (CSPG), which may be an important inhibitor of axonal migration and regeneration (Ridet et al., 1996; Silver and Miller, 2004), laminin an important element for neuronal migration and neurite extension (Ridet et al., 1996; Costa et al., 2002), and matrix metalloproteases (MMP) important for modulating the extracellular environment and can prove essential for neuronal survival (Gasche et al., 2006). Investigators primarily use three approaches to dissect the contribution of increased ECM in gliosis and reduce its formation *in vitro*. The first is interruption of ECM synthesis, the second is enzymatic or chemical degradation of ECM, and the third is the use of antibodies to block the function of ECM (McGraw et al., 2001).

Up-regulation of ECM in response to TBI has been verified using immunofluorescent techniques (Fitch and Silver, 1997). A qualitative increase in staining of labeled CSPG was observed with the expression pattern was restricted to an area of direct tissue damage and the area immediately adjacent (Fitch and Silver, 1997). However, an increase in CSPG expression did not always coincide with GFAP expression but did coincide with the breakdown of the BBB and was associated with inflammatory infiltrates. If the BBB remained intact after injury the ECM levels remained comparable to controls (Canning et al., 1996). Suggest that disruption of the BBB is necessary for the up-regulation of ECM molecules.

ECM synthesis interruption by the use of beta-D-xyloside, a specific proteoglycan synthesis inhibitor, has been demonstrated to reduce CSPG *in vitro* (Fichard et al., 1991). Under this paradigm, it was shown that astrocytes became more permissive for neurite outgrowth (Smith-Thomas et al., 1995; McGraw et al., 2001). Enzymatic degradation has been used to asses the deposition of ECM and evaluate the subsequent regeneration of neurons through it. One illustration of this is the treatment of glial scar explants with chondroitinase, an enzyme which selectively cleaves GAG side chains of CSPGs. This model showed that cultured retinal ganglion cells could only extend their neurons after the treatment (McKeon et al., 1991; Silver and Miller, 2004). A representation of the use of antibodies to inhibit the function of the ECM is treatment of lesioned neuron-astrocyte mouse cortical co-cultures with antibodies which recognized the astroglial laminin. This assay resulted in an inhibition of both neuronal access to the lesion site and neurite outgrowth, suggesting an active role for laminin in ability of axons to regenerate in the CNS after injury (Costa et al., 2002). Thus suggesting that laminin and laminin bioavailability, subsequent to a reduction in astrogliosis, may be important permissive elements for neuronal migration and neurite outgrowth (Costa et al., 2002; Silver and Miller, 2004).

CNS injury studies involving cerebral ischemia and intracerebral hemorrhage have demonstrated astrocyte derived MMPs, including astrocyte matrix metalloprotease 2, which can digest components of the blood–brain barrier matrix and contribute to the physical disruption of the blood–brain barrier thus leading to edema and to the influx of leucocytes into the CNS (Mun-Bryce and

Rosenberg, 1998; Rosenberg et al., 1998; Rosenberg et al., 2001; Gasche et al., 2006). MMPs ability to digest the basal lamina of capillaries increases the risk of hemorrhagic transformation of the ischemic tissue (Gasche et al., 2006).

Alternatively, gliosis may not have a consistent negative influence on recovery from ischemia. Glial cells surrounding axons have a key role in determining CNS regenerative capacity (Sivron et al., 1993). It has been reported that during ischemia and hemorrhage of the CNS, multiple MMPs and serine proteases are produced along with their inhibitors (Mun-Bryce and Rosenberg, 1998). Among the former, MMPs are involved in the breakdown of the ECM component. This breakdown has been shown increase the availability of laminin and thus may be important permissive elements for neuronal migration and neurite outgrowth (Costa et al., 2002). In addition, during the acute ischemic phase, maintenance of the ECM is essential for neuronal survival (Gasche et al., 2006).

On the other hand, lack of CNS regeneration following injury/neurotrauma has been an area of intense research over the past decade. It is well accepted that the formation of reactive gliosis associated ECM molecules interfere with neural repair or CNS axonal regeneration (Ridet et al., 1997; Steeves and Tetzlaff, 1998). Additionally, ECMs contribute to the formation of the glial scar, its permissiveness of neuronal repair, and their extension of neurites into the lesioned area (McKeon et al., 1991). This evolutionarily conserved function of reactive astrogliosis in response to CNS injury may be primarily to isolate the lesion area to prevent further expansion of inflammation. This “sequestration” has

been shown to be an impediment to regenerating axons (Smith et al., 1986; McKeon et al., 1991; Bush et al., 1999). In addition, reactive astrocytes up-regulate the synthesis of collagens and sulfate proteoglycans, which also can impede neurite growth (Fichard et al., 1991; Smith-Thomas et al., 1995; Ridet et al., 1997).

In summary, 3 of the most studied ECMs are CSPGs, laminin, and MMPs (Ridet et al., 1996; Costa et al., 2002; Silver and Miller, 2004; Gasche et al., 2006). Three approaches commonly used to dissect the contribution of ECM in gliosis are synthesis interruption, degradation, antibody interference of function (McGraw et al., 2001). In response to injury up-regulation CSPG was restricted to the injured area and associated with breakdown of the BBB and increased inflammatory infiltrates, but its expression did not always coincide with GFAP expression (Fitch and Silver, 1997). Its synthesis interruption and enzymatic degradation revealed an increase in neurite outgrowth (Smith-Thomas et al., 1995; McGraw et al., 2001). CVA studies of MMPs can add to the disruption BBB (Mun-Bryce and Rosenberg, 1998; Rosenberg et al., 1998; Rosenberg et al., 2001; Gasche et al., 2006) but can also increase the availability of laminin and enhance neuronal migration and neurite outgrowth (Costa et al., 2002). But ECM molecules can contribute to glial scar formation (Smith et al., 1986; McKeon et al., 1991; Bush et al., 1999) do interfere with neural repair or CNS axonal regeneration (Fichard et al., 1991; Smith-Thomas et al., 1995; Ridet et al., 1997; Steeves and Tetzlaff, 1998).

Functional Hallmarks of Reactive Astrocytes

Reactive astrocytes play many functional roles in the mediation of CNS damage and recovery (Aschner et al., 2002). The most significantly investigated areas are inflammation, excitotoxicity, edema, blood brain barrier maintenance, and formation of the glial scar (Sofroniew, 2005). The orchestration of these events has been postulated to minimize damage and provide maximum benefit to the organism (Seifert et al., 2006).

Inflammation

To investigate reactive astrocyte modulation of inflammation, a transgenic mouse model that express a glial fibrillary acidic protein-herpes simplex virus-thymidine kinase transgene which selectively targets to reactive astrocytes using the GFAP promoter (GFAP-HSV-tk) was used (Holland and Varmus, 1998). Treatment of these mice with the antiviral agent, gancyclovir, conditionally ablates proliferating reactive astrocytes (Bush et al., 1999). In response to TBI of different severities (Myer et al., 2006) immunohistochemical evaluation and quantitative morphometry of moderate or severe injury in forebrain tissue was reported. Moderate TBI in control mice triggered extensive and persisting reactive astrogliosis, with most neurons being preserved, little inflammation and an 18% loss of cortical tissue beneath the impact site. Ablation of reactive astrocytes after moderate TBI in transgenic mice caused substantial neuronal degeneration and inflammation, with a significantly greater 60% loss of cortical tissue. However, severe TBI in both the ablated GFAP-HSV-tk model and control

mice revealed similar results which were pronounced neuronal degeneration and loss of about 88% of cortical tissue (Myer et al., 2006). Similar results were gleaned with the same populations of mice using another form of TBI (Bush et al., 1999). It was reported that in mice lacking the reactive astrocytes, a small stab or spinal cord crush injury resulted in leukocyte infiltration, local tissue disruption, severe demyelination, neuronal and oligodendrocyte death, pronounced motor deficits and failure of BBB to reform (Faulkner et al., 2004). Additionally, moderate crush injuries caused widespread tissue disruption, pronounced cellular degeneration, and failure of wound contraction, with severe persisting motor deficits (Faulkner et al., 2004). It was also noted that leukocytes gained increased and prolonged entry into the CNS parenchyma.

In response to damage, reactive astrocytes can release inflammation factors that initiate responses in target cells. These responses are similar to the responses of activated immune cells in the periphery such as leukocyte infiltration (Streit et al., 2004). However, this infiltration can only be accomplished via destruction or compromise of the blood-brain barrier (Streit et al., 1998; Sroga et al., 2003; Streit et al., 2004).

One such factor is CCL20. This molecule has been identified as a chemoattractant for immature dendritic cells and memory/differentiated T-cells and B-cells. Strong immunohistochemical evidence has demonstrated that astrocytes represent the main CNS source of CCL20 which can be released into the environment in response to damage (Ambrosini et al., 2003). It was further established *in vitro* that CCL20, harvested from the supernatants from cytokine-

activated astrocytes, has the ability to stimulate the migration of polarized T-cells from the periphery, and contribute to the inflammatory response (Ambrosini et al., 2003). Evidence shows that astrocytes could play an important role in orchestrating the recruitment of specific leukocyte subsets to the inflamed CNS and in regulating CNS-targeted immune responses.

In response to inflammation reactive astrocytes produce large amounts of prostaglandins (PGs) (Clemens et al., 1996; Minghetti and Levi, 1998). Studies show they also respond to the pro-inflammatory cytokine IL-1 with an increase in the production of PGs that is due to upregulation of secretory phospholipase-2 and cyclooxygenase-2 (COX-2) (Dayton and Major, 1996; O'Banion et al., 1996; Luo et al., 1998; Molina-Holgado et al., 2000). The proinflammatory cytokine interleukin-1 (IL-1) is induced early after brain injury and has been implicated in the delayed damage. To further investigate this, a transgenic mouse model which overexpresses the human secreted form of IL-1ra, a IL-1 receptor antagonist was created (Yang et al., 1997). This expression is targeted to astrocytes by the murine glial fibrillary acidic protein promoter. The IL-1ra has been shown to modulate the proinflammatory cytokine cascade by blocking the binding of IL-1 to its signaling receptor (Yang et al., 1997). In response to TBI, following an acute inflammatory response, the neurological damage was significantly higher in the transenic mice as compared to wild types (Tehranian et al., 2002). Further cytokine expression showed significantly increased levels of TNF alpha IL-1beta and IL-6 in the cerebral cortex from the wild type mice. The transgenic mice revealed no significant changes of TNF alpha IL-1beta and IL-6 levels. Thus

blocking IL-1 signaling by elevated levels of IL-1ra has a neuroprotective effect in response to injury (Tehranian et al., 2002).

In summary, these findings demonstrate the protective nature of reactive astrocytes. They provide essential activities and preserve function after injury. Ablation of dividing reactive astrocytes exacerbated inflammation, cortical degeneration and healing abnormalities following moderate TBI, but did not alter cortical degeneration after severe TBI. CCL20 released from astrocytes can stimulate the migration of polarized T-cells from the periphery, and contribute to the inflammatory response (Ambrosini et al., 2003). These findings indicate that the reactive astrocytes play essential roles in preserving neural tissue and restricting inflammation after moderate focal brain injury.

Excitotoxicity

Glutamate is the most abundant excitatory neurotransmitter in the CNS (Hardman et al., 2001). Following CNS injury, extracellular levels of glutamate can increase acutely (Faden et al., 1989; Palmer et al., 1993; Globus et al., 1995) causing over-stimulation of glutamate receptors, in particular, the NMDA receptors (NMDAR). This overactivation of glutamate receptors is known as “excitotoxicity” (Olney, 1969, 1990). The glutamate efflux resulting from TBI can emanate from different sources. Glutamate may move into brain via blood flow following disruption of the BBB (Koizumi et al., 1997). It has been reported that following TBI intraparenchymal hemorrhage is often seen. Autoradiographic examination reveals marked extravasation following intravenous injection of ^{14}C-labeled glutamate at the site of cortical impact (Koizumi et al., 1997).

Leakage from damaged cells has also been shown to lead to glutamate overflow into extra-synaptic regions. This leakage may also reflect non-specific development of membrane micropores in the cell membrane. This is based on the finding that the release of excitatory amino acids is closely related to the release of structural amino acids (Bullock et al., 1998; Yi and Hazell, 2006).

Decreased glutamate transporter function may also contribute to the accumulation of extracellular glutamate. This dysfunction, due either to functional impairment of the transporter structures or decreased expression of the transporter proteins, can contribute significantly to the accumulation of extracellular glutamate. Decreased transporter activity has also been hypothesized to contribute to this excess (Yi and Hazell, 2006). Another theory is that glutamate release may result as a consequence of glutamate transporter reversal. However, this has not been proven in TBI, it has been reported under conditions of ischemia (Gemba et al., 1994).

Excitotoxicity may also reflect excessive synaptic release subsequent to the damage (Hardman et al., 2001). This critical release could lead to glutamate overflow into extrasynaptic regions. Recent studies following TBI have identified a transient up-regulation of two nerve terminal proteins that are intimately associated with the synaptic vesicle release machinery of the cell, complexin I and complexin II (Yi and Hazell, 2006). These proteins are localized in inhibitory and excitatory synapses respectively, and are considered to play an significant function in the modulation of neurotransmitter release and the maintenance of normal synaptic function (Pabst et al., 2000; Hu et al., 2002). Intriguingly,

following brain trauma, up-regulation of complexin II has been reported, suggesting it may be an important contributing factor to increased extracellular levels of glutamate following injury (Yi and Hazell, 2006). Other studies have shown that protein reduction can contribute to excitotoxicity. Reports indicate that TBI induces a transient down-regulation of glial glutamate transporter proteins (GLT-1 and GLAST) (Rao et al., 1998). The protein expression of GLT-1, GLAST, and neuronal glutamate transporter EAAC1 in response to a transient focal cerebral ischemia generated by MCAo has also been examined. 72 hours of reperfusion after transient MCAo significantly decreases GLT-1 and EAAC1 protein levels. A significant decrease of the GLT-1 and EAAC1 mRNA expression at both 24 hour and 72 hours of reperfusion (Rao et al., 2001) has also been demonstrated. This evidence supports the hypothesis that reduced astrocyte function can contribute to excitotoxicity.

In summary, increases in available glutamate emanating from the disruption of the BBB, leakage from damaged cells, altered glutamate transporter function, excessive synaptic release, dysregulation of synaptic release mechanisms can result in excitotoxicity and thus exacerbate damage to the CNS following injury.

Edema

Two types of edema have been shown to contribute to the increase in brain tissue volume. The first is vasogenic, in which water enters the brain as a result of a BBB compromise and thus accumulates in the extracellular space, and second is cytotoxic, in which water enters cells causing them to swell (Klatzo, 1967). Ablation studies reveal that astrocytes play a significant role to the

development of brain swelling or edema (Bush et al., 1999; Kimelberg, 2005). Current thought ascribes the regulation of water homeostasis to two astrocytic attributes, the first being their end-feet channels, which modulate the flow of water and solutes across the BBB, and the second being gap junctions, which connect the cytoplasmic environment of adjacent astrocytes (Beaumont et al., 2000).

Water channels named aquaporins (AQPs) play an significant role in astrocyte swelling the subsequent cerebral edema formation (Venero et al., 2001). These water-selective channels belong to a family of membrane proteins that mediate rapid transmembrane transport of water. AQP have been shown to be the primary route by which water moves in and out of astrocytes in response to osmotic changes (Venero et al., 2001; Agre, 2004). Twelve members of the aquaporin family have been identified and of special interest is AQP4. AQP4 is expressed in astrocyte foot processes and ependymocytes facing capillaries and brain-cerebrospinal fluid (CSF) interfaces (Badaut et al., 2002). Genetic knockout of AQP4 in mice demonstrates less astrocyte swelling, reduced brain edema, and better neurologic outcome after focal ischemia (Manley et al., 2004). The results support the AQP4 function in these processes. Furthermore, up-regulation of AQP4 on reactive astrocytes has been reported after ischemia in mice (Vizuete et al., 1999; Badaut et al., 2001; Chen and Swanson, 2003). Up-regulation of AQP4 expression following TBI has also been reported in humans.

Evidence indicates that brain AQP4 water channels are modulated by vasopressin V1a receptors (Shuaib et al., 2002; Kleindienst et al., 2006). Thus

the edema-reducing properties of the selective V1a receptor antagonist, SR49059, following MCAo were examined (Kleindienst et al., 2006). Male rats were infused with SR49059 infusion at different dosages and timing intervals before or after MCAo. After a 2-hour period of ischemia and 2 hours of reperfusion, the animals were sacrificed for assessment of brain water content, sodium, and potassium concentration. It was reported that SR049059 treatment reduced brain water content in the lesioned area. This treatment prevented the subsequent influx of sodium to the brain while the potassium loss was inhibited. These findings imply that in ischemia induced brain edema, inhibition of AQP4 can modulate brain edema and subsequent sodium shift into the brain (Kleindienst et al., 2006). This supports the suggestion that water entry into cells through AQP4 may be detrimental in which cytotoxic brain edema is predominant.

It has also been proposed that AQP4 may function to clear excess water from the brain, thereby decreasing vasogenic edema and intracranial pressure (Manley et al., 2004). Using a TBI injury model, it was demonstrated injury decreased AQP4 expression level in the injury core and modestly increased it in the penumbra (Zhao et al., 2005). Post injury administration of sulforaphane, an isothiocyanate, attenuated AQP4 loss in the injury core and further increased AQP4 levels in the penumbra region compared with injured animals receiving vehicle. The increases in AQP4 expression were accompanied by a reduction in brain edema. Thus suggesting that AQP4 in necessary for water clearance from the damaged area.

Astrocytes are extensively networked together by small transmembrane pores called gap junction channels. Gap junction channels are hemi-channels that when located opposed to one another form a cell-cell channel which can directly link the cytoplasm of the opposing cells. These hemi-channels are found in most cell types in vertebrates and permit the passage of ions and molecules of less than 1 kDa. Gap junction dysfunction may exacerbate cell injury, mediate paracrine, or autocrine signaling by the propagation of cell damage and death signals through the channel openings within affected tissues (Contreras et al., 2004).

Studies reveal astrocytes can assemble channels via two classes of gap junctions; those that are composed of homomeric Cx26 channels and those composed of Cx43 - Cx30 heteromeric channels (Altevogt and Paul, 2004). These gap junctions under normal circumstances mitigate regional homeostasis (Anderson et al., 2003; Kielian and Esen, 2004) and have been hypothesized to contribute to ischemic cell death by the diffusion of small pro-apoptotic substances through the channels from the core to the penumbra (Lin et al., 1998).

Gap junctions may contribute to cytotoxic edema by allowing ATP to flow through these channels from healthy to dying cells and thus may strip the region bordering the injured area from energy rich phosphates (Lin et al., 2003; Anderson et al., 2004). Other evidence suggests that gap junction coupling may directly contribute to the propagation of spreading depressions, promote the expansion of the damage and thus contribute to edema (Nedergaard, 1994;

Anderson et al., 2004). In support of this, animals were pretreated with the gap junction blocker, octanol, before MCAo and sizes of the ischemic lesions were compared to those that received only the vehicle. The octanol-treated animals showed a significantly decreased mean infarction volume as compared to controls (Rawanduzy et al., 1997).

In summary, astrocyte associated aquaporins and gap junctions have been shown to contribute to CNS edema following injury. Knockout of AQP4 demonstrated less astrocyte swelling, reduced brain edema, and better neurologic outcome after focal ischemia (Manley et al., 2004). Its up-regulation has been reported after ischemia (Vizuete et al., 1999; Badaut et al., 2001; Chen and Swanson, 2003) and TBI. Inhibition of AQ4 reduced brain water content in a lesioned area and the influx of sodium to the brain while the potassium loss was inhibited. Alternately, this bi-directional channel has been reported as necessary for water clearance from damaged areas. Thus implying that inhibition of AQP4 can modulate brain edema and subsequent sodium shift into the brain (Kleindienst et al., 2006). Astrocyte associated gap junction channels may contribute to cytotoxic edema by allowing ATP to flow from healthy to dying cells. They may also contribute to the propagation of spreading depressions, promote the expansion of the damage and thus contribute to edema (Nedergaard, 1994; Anderson et al., 2004) Their inhibition in concert with MCAo showed a significantly decreased mean infarction volume as compared to controls (Rawanduzy et al., 1997) thus supporting this argument.

Blood Brain Barrier

Transgenic studies have clearly demonstrated many roles of reactive astrocytes in the repair of the BBB. It has been demonstrated that reactive astrocytes can promote repair of the blood-brain barrier through production of extracellular matrix components (Kakinuma et al., 1998). Studies including the GFAP-HSV-tk model with targeted ablation of reactive astrocytes after stab injury showed that it prevented BBB repair and promoted vasogenic edema (Bush et al., 1999). Studies involving the GFAP $^{-/-}$ and GFAP$^{-/-}$ VIM$^{-/-}$ animal models have also revealed a significantly prolonged healing time of the BBB, disruption of debris clearance of the wound site and a higher proportion of the animals died quite possibly due to bleeding within the injured area (Pekny et al., 1999).

In summary astrocyte modulation of the BBB in response to injury indicates they serve to restore the BBB and thus, prevent an overwhelming inflammatory response, limit cellular degeneration and reduce edema. The significant prolonged healing period for animals lacking astrocytes or IF proteins and disruption of debris clearance support this posit.

Glial Scarring

The scar formation process is not completely understood. However, a rough timeline can be established. Minutes after injury, neurons and glial cells undergo cell death. Astrocytes, microglia, and monocytes are recruited to the site (Dusart and Schwab, 1994). If the damage penetrates the dura matter, meningeal cells and fibroblasts also migrate into the wound site and assist in the reformation of

the basal lamina (Carbonell and Boya, 1988). GFAP and VIM are up-regulated in resident astrocytes (Eng and Lee, 1995). Nestin expression within the injured site is also induced in reactive astrocytes (Frisen et al., 1995). Reactive astrocytes begin to sequester the lesion area within 3 to 5 days post injury (Hatten et al., 1991) by interdigitation of their processes to form the scar. The sum of these actions forms dense plexus of astroglial cells which isolate the damaged portion from the healthy adjacent area of the CNS. This process is known as anisomorphic gliosis (Malhotra et al., 1993; Bignami and Dahl, 1995; Ridet et al., 1996). Isomorphic gliosis is a process in which there is a failure of the glial scar to form. This occurs in areas distal to the lesion site where the cytoplasmic architecture has been less disturbed (Malhotra et al., 1993; Ridet et al., 1996; McGraw et al., 2001).

Examination of the CNS reaction to TBI in $GFAP^{-/-}$, $VIM^{-/-}$, or $GFAP^{-/-}VIM^{-/-}$ mice models revealed apparent normal glial scar formation after spinal cord or brain lesions in $GFAP^{-/-}$ or $VIM^{-/-}$ mice, but this formation was significantly impaired in $GFAP^{-/-}VIM^{-/-}$ mice. These mice developed less dense scars which were often accompanied by bleeding. The scar tissue seen in these of these animals was interrupted by numerous fissures most often running in a dorsal to ventral orientation. These fissures often were filled with blood, tissue fluid or debris. Injured animals with a 2 week chase period following injury had large numbers of red blood cells within the scar tissue. This suggests a more pronounced bleeding period and/or defective clearance of blood from the damaged site (Pekny et al., 1999; Ribotta et al., 2004).

Ablation of astrocytes by injection of ethidium bromide, which transiently kills dividing cells by binding to cellular DNA and RNA, into the lesion site showed an inhibition of glial scar formation while astrocytes were absent and its reappearance at their return (Moon et al., 2000). This work was supported by astrocyte ablation by gancyclovir treatment in the GFAP-HSV-tk mouse which also showed failure of the glial scar to form which was accompanied by pronounced interstitial edema, numerous blood born inflammatory and phagocytic cells and enhanced neurodegeneration (Bush et al., 1999).

The identification of reactive astrocytes surrounding dystrophic non-regenerating neuronal fibers supported the hypothesis that reactive astrocytes are the causative factor for failed regeneration (Silver and Miller, 2004). Transgenic work has subsequently verified that the obstruction of neurite extension can be attributed to the formation of the glial scar. Work involving the use of the GFAP-HSV-tk model in response to stab injury of the forebrain or spinal cord, resulted in an increase in axon regeneration within the lesioned area in the reactive astrocyte ablated mice as compared to controls (Bush et al., 1999). In the double knock out GFAP$^{-/-}$ VIM $^{-/-}$ mouse model mentioned above, a significant extension of neurites was also reported in response to similar injury (Menet et al., 2000; Menet et al., 2001; Menet et al., 2003). Furthermore, IFs have also been shown to contribute to a partitioning effect of the glial scar for axonal extension (Silver and Miller, 2004). *In vitro* studies of astrocytes cultured from GFAP$^{-/-}$ mice or treated with GFAP antisense DNA demonstrate increased expression of laminin and thus these areas become more permissive to neurite

growth (Bush et al., 1999; Menet et al., 2000; Menet et al., 2001; Costa et al., 2002; Menet et al., 2003; Silver and Miller, 2004).

In summary, glial scarring is the result of a rapid response from resident astrocytes to traumatic injury of the CNS. The functional role, on one hand, is suggested protective in nature, in that it is thought to be an attempt made by the CNS to restore homeostasis and prevent further damage from secondary lesions through isolation of the damaged region (Ridet et al., 1997). This has been demonstrated by transgenic models. Ablation studies reveal increases in damage with the loss of reactive astrocytes. Furthermore GFAP and VIM are required for proper glial scar formation in the injured central nervous system and that some degree of functional overlap exists between these IF proteins. On the other hand, the formation of this structure is thought to be a negative modulator of axon growth following adult CNS TBI and CVA (Fitch and Silver, 1997). It's inhibitory nature to the repair and regeneration of injured neurons by the production of a non-permissive substrate for axonal regeneration (Bahr et al., 1995).

Heterogeneity of Reactive Astrocyte Response

Reactive astrocytes response to damage varies according to age of the organism and location of the astrocyte in the CNS. Analysis of astrogliosis variations between adult and neonatal organisms reveals a more vigorous reactive gliosis in adult than in neonatal animals (Ridet et al., 1996). Furthermore, there is a gradient of intensity of response that positively correlates with distance from the injured site. Proximal reactive astrocytes show increased

expressions of IFs, dehydrogenases, neurotrophic factors, cytokines, and cell surface molecules, while distal reactive astrocytes display only moderate expression of these proteins (Malhotra et al., 1993; Ridet et al., 1996). Astrocytes also do not respond in a stereotypic manner to all forms of CNS insult (Ridet et al., 1996). Studies have revealed that reactive gliosis is a heterogeneous response that varies qualitatively and quantitatively depending on both the nature of the infliction and the microenvironment of the disturbed site. Different categories of CNS lesions can produce reactive astrocytes that are biochemically heterogeneous and can be subclassified by immunocytochemical screening procedures (Eddleston and Mucke, 1993; Malhotra et al., 1993; Ridet et al., 1996).

Extensive research has confirmed that astrocytes are not homogenous. Their variance may be due to the location in which they reside in the CNS. An example of this variance was determined the concentration of soluble GFAP and the specific activity of glutamine synthetase (GS) in eleven CNS regions in the adult rat (Patel et al., 1985). This study revealed significant regional distribution differences in these astrocyte marker proteins and a five fold difference in ratio of protein expression which was CNS location dependent. Further reports indicate that immunochemical detection of GS and GFAP in whole homogenates of different regions were due to the differences in their absolute protein concentrations (Patel et al., 1985). This gradient of characteristics may reflect anatomical environmental influences and may be responsible for the differences in response to CNS insult. Moreover, controversy exists as to whether the

disparate response of astrocytes is due to the existence of a subpopulation of astrocytes. One theory is there are “reaction prone” astrocytes or astroblast in the CNS. However, this has yet to be proven. This diversity of response seems to be a modulated reaction to the different microenvironmental conditions, with respect to combinations of growth factors, cytokines, adhesion molecules, serum factors, and other signals produced by injured neurons, microglia, endothelial cells, and from components of extravasated serum (Malhotra et al., 1993; Ridet et al., 1996).

Reactive Astrocyte Detrimental and Beneficial Roles Summary

The utility of reactive gliosis cannot be understated. Various target mutations which selectively abolish reactive astrocytes have clearly revealed its significance and beneficial roles (Pekny et al., 1995; Bush et al., 1999; Pekny et al., 1999; Sofroniew et al., 1999; Faulkner et al., 2004; Ribotta et al., 2004). The $GFAP^{-/-}VIM^{-/-}$ transgenic mice clearly demonstrate that reactive astrocytes demarcate an injured area by the formation of the glial scar, and restrict the inflammatory response in the injured CNS environment. They also stimulate BBB repair counteract edema and modulate blood flow (Eliasson et al., 1999). Furthermore, ablation of reactive astrocytes in the injured CNS is associated with a significant increase in neuronal degeneration, prolonged infiltration leucocytes, and to failure of the blood-brain barrier to re-establish itself following injury. Their detrimental role, inhibition of axonal regeneration is also supported the loss of reactive astrocytes. Ablation of reactive astrocytes is associated with an enhanced increase in local neurite outgrowth and possible regeneration of the

CNS (Malhotra et al., 1990; Bush et al., 1999; Sofroniew, 2005). Understanding the mechanisms behind these events and their impact can lead the way for therapeutic intervention to ameliorate damage and promote recovery to the adult CNS.

Astrocytes as Neural Stem Cells in the CNS

Introduction Astrocytes as Adult Neural Stem Cells

The doctrine of an immutable adult brain is a result of numerous early observations. The most notable were from Koelliker, His, and Ramón y Cajal (Koelliker, 1896; His, 1904; Ramón y Cajal, 1999; Gross, 2000). Using the techniques of their time, they meticulously described the recurring appearance, and invariable architecture of the adult CNS and the differentiated structure of the neuron. This unalterable network of structures coupled with developmental studies lent credence to the belief that the brain was completely formed by adulthood. Moreover, no evidence existed at the time to either support the possibility of neuronal proliferation following brain injury, or neural mitosis in the brains of adult birds and mammals (Altman and Das, 1965). Thus, this belief scripted the dogma of neuroscience that has lasted for well over 100 years “that no new neurons are added to the adult mammalian brain”. This dogma has also caused the prejudicial dismissal of evidentiary figures of mitotic and migratory structures because the techniques of the time lacked the ability to definitively scrutinize the structures (Gross, 2000).

Advances of technology and science over the last one hundred years have pushed our ability to visualize and thus understand the dynamic adult mammalian brain. One interesting field is the repair process of the adult brain. Another field yet to be explored is the mechanisms of cellular turnover in the CNS and how these replacement cells maintain or augment cognitive function. It is now established that there is neuropoiesis in the adult brain (Emsley et al.,

2005) and the suggestion that disruption of this neurogenesis may lead to CNS damage and disease (Handler et al., 2000; Scharfman et al., 2000; Curtis et al., 2003; Jin et al., 2004a; Jin et al., 2004b). Several studies have proposed that astrocytes may contribute to neurogenesis as a source of trophic substances regulating it. Strong emergent evidence supports the hypothesis that astrocytes also act as neural stem cells (NSCs) in the CNS. This review will detail evidence supporting the role of astrocytes as adult neural stem cells.

Criteria for Stem Cell Identification

Stem cells have been characterized as unspecialized human or animal cells that have the capacity to generate multiple types of differentiated cells and at the same time maintain their ability for self-renewal (Morrison et al., 1997). Other properties attributed to stem cells have comprised of the ability to undergo asymmetric cell division, exist in a mitotically quiescent form, and clonally regenerate all of the different cell types that constitute the tissue in which they exist (Hall and Watt, 1989; Potten and Loeffler, 1990; Morrison et al., 1997).

In the mid 1960's, it was demonstrated the ability of certain regions of the adult rat brain to continually add new neurons. This was performed by labeling dividing cells with an injection of thymidine-H^3 and visualizing the results using autoradiography (Altman, 1962b, 1962a, 1963; Altman and Das, 1965). Because of the scientific climate, this work existed largely uncelebrated. But attitudes matured near the turn of the century and this work was revisited. It was later hypothesized in that these new neurons were derivatives from a population of adult neural stem cells (NSCs). A series of experiments ensued to isolate the

putative stem cells. The first successfully recognized work came in 1992, when it was demonstrated that neural stem cells taken from the adult murine brain can be propagated *in vitro* (Reynolds and Weiss, 1992). These cells also maintained the capacity for self-renewal and could generate the three major classes of CNS cell types; neurons, astrocytes, and oligodendrocytes in response to epidermal growth factor (EGF). Furthermore, the cells with neuronal morphology were immunoreactive for gamma-aminobutyric acid and substance P, two neurotransmitters of the adult striatum *in vivo* (Reynolds and Weiss, 1992). It was also demonstrated that NSCs could be isolated from the walls of the ventricular system and from the hippocampus of the adult rodent CNS (Lois and Alvarez-Buylla, 1993; Morshead et al., 1994; Weiss et al., 1996; Palmer et al., 1997). During this time it was reported that stem cells associated with the lateral ventricles of the brain give rise to immature neurons which travel along the rostral migratory stream (RMS) to the olfactory bulb, where they differentiate and integrate as interneurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Johansson et al., 1999a). It was further demonstrated that, in adult humans, both the lateral ventricle wall and the hippocampus harbor self-renewing cells capable of generating neurons, astrocytes, and oligodendrocytes *in vitro* (Johansson et al., 1999b).

Brain Marrow

In an effort to understand and characterize the neurogenic regions of the brain researchers have introduced the concept of a “brain marrow” (Scheffler et al., 1999). A place in which pluripotent stem cells and developmentally regulated

molecules are located within a central core of the mature brain to provide a continuum of neuropoiesis in a manner which parallels hematopoiesis of the bone marrow. The two areas with the most convincing evidence of neuropoiesis are the region associated with the lateral ventricle and the proliferative region associated with the hippocampal dentate gyrus. These areas have been subsequently identified as the subventricular zone (SVZ) and the subgranular zone (SGZ), respectively (Hagg, 2005). These germinal regions were once associated with a core of proliferation of the CNS in development. Other groups have also reported neural stem cell capabilities in other CNS locations (Palmer et al., 1995; Arsenijevic et al., 2001; Seri et al., 2006).

Recent evidence for a new site for murine NSCs has been reported (Seri et al., 2006). This site has been identified as the subcallosal zone (SCZ). It is the caudal extension of the SVZ which is no longer associated to an open ventricle. It lies between the hippocampus and the corpus callosum and is assumed to be the vestigial remnant of the proliferative germinal matrix of the embryonic forebrain. Enlargement of the hippocampus during development closes off this once ventricularly associated area however isolated patches of ependymal cells remain in this area. Cells isolated from the SCZ and subsequently cultured as neurospheres demonstrated NSCs behavior in vitro. Retroviral labeling and homotypic-homochronic micro-transplantation techniques demonstrate that the majority of cells born in the SCZ migrate into the corpus callosum to become oligodendrocytes in vivo. However unambiguously identification of which cells type in the SCZ corresponded to the progenitors that gave rise to the cultured

neurospheres was not possible under this paradigm. However, the possibility that the progenitors of the SCZ could be astrocytes was not ruled out (Seri et al., 2006).

Advances in the Identification of Neural Stem Cells

Enhancement of cell culture techniques invited the use of growth factors. The first widely accepted was EGF as mention above, the second was basic fibroblast growth factor (bFGF). bFGF growth factor was able to stimulate the proliferation of neuronal and glial progenitors isolated from the septum and striatum of adult rats *in vitro* (Palmer et al., 1995). It was reported that the generated progenitors initially harvested from the septum and striatum were indistinguishable from those isolated from the adult hippocampus and subventricular zone, two regions that generate neurons well into adult life. Although a variety of cell types are initially isolated from each brain region, the progenitor-like cells from all four regions studied were capable of considerable proliferation. These cells with limited serial passage can be cultured as enriched populations of immature cells that are capable of differentiating into mature glia and neurons following density arrest and growth factor withdrawal. It was also reported that cells isolated from the septum and striatum proliferate and have the ability to differentiate into neurons once they are removed from their local environment (Palmer et al., 1995). This result may indicate that neurogenesis may be restricted to discrete areas of the adult brain by regional differences in regulatory signals rather than from an absence of progenitors capable of responding to neurogenic cues (Palmer et al., 1995).

Neural Stem Cell Type and Description

Cell Type	Description	Corresponding Cell Type
A	Migrating Neuroblasts	
B	Slowly Proliferating Astrocytes	Type 1 cells
C	Rapidly Dividing Immature Precursors	
D	Transient Neuronal Precursors	Type 2 cells

Figure 1

A topographical model for *in vivo* identification of stem cells in the adult mammalian brain has been established (Doetsch et al., 1997). Three cell types involved in the generation of olfactory bulb neurons as Type A, B and C cells have been described. Type A cells have the ultrastructure of migrating neuronal precursors. These cells advance as chains through tubes defined by processes of slowly proliferating astrocytes, and are immunoreactive for polysialylated neural adhesion cell molecule (PSA-NCAM), TuJ1 (tubulin), and nestin but not GFAP or vimentin. Type B cells ensheath chains of Type A cells and demonstrate immunoreactivity for nestin, GFAP, and VIM but not PSA-NCAM and TuJ1. Type A and B2 (but not B1) cells incorporated thymidine-H^3. Type C cells are the most actively dividing cell in the SVZ. These cells have an immature ultrastructural character and are nestin positive but negative for other markers describe above. Type C cells form focal clusters closely associated with chains of Type A cells but are not found in the RMS (Doetsch et al., 1997).

Advancement for development neuronal precursor identification came from development of culturing methods. Reproducible clonal cell culturing method to

isolate neuronal progenitors has been established (Reynolds and Weiss, 1996). In this method, primary cultures are treated with EGF in the absence of poly-cation substrates. This produces rapid proliferation and free floating neurospheres of undifferentiated cells. The differentiation of single primary sphere by dissociation, removal of EGF, plating on poly-cationic substrates, and exposure to fetal bovine serum (FBS), results in the production of neurons, astrocytes, and oligodendrocytes. However, if the neurospheres are dissociated into single cells and subsequently plated after serial dilution as 1 cell/well, in the presence of EGF, and in the absence of poly-cation substrates, they generate clonally derived secondary spheres. Differentiation of single secondary spheres by removal of EGF and addition of FBS results in the production of neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss, 1996). This is the most common method for identification of neural precursors.

Astrocytes can Control Neural Induction of Stem Cells

Adult astrocytes can induce a neuronal fate specification in the hippocampus. This work was performed using clonal derived neural stem cells isolated from the hippocampus of adult rats which were retrovirally engineered to express green fluorescent protein (GFP). Hippocampal astrocytes actively regulate adult neurogenesis both by instructing neuronal fate commitment and by promoting proliferation of adult neural stem cells. This influence was mediated by both diffusible and membrane-bound factors originating from astrocytes. Hippocampal progenitor cells in this study were GFAP negative, leaving open the possibility of a novel progenitor. Astrocyte influence is also regionally specified: astrocytes

harvested from the adult hippocampus maintain the potential to promote neurogenesis, while this potential is lost in astrocytes from adult spinal cord. Neuronal production in the mature brain is regulated, at least in part, by the regional properties of astrocytes. These results suggest together with reports of astrocyte regulation of synapse formation and synaptic transmission support the belief that astrocytes have an active regulatory role in the maintenance of the CNS (Song et al., 2002).

The Origins of Neural Stem Cells

Subependyma and Neural Stem Cell Origin

It was reported that ependymal cells were neural stem cells (Johansson et al., 1999a). In order to demonstrate this, specific labeling of cells in the ependymal layer throughout the neuraxis by injecting either the fluorescent label DiI or a replication-deficient adenovirus expressing lacZ into the ventricular lumen was performed (Johansson et al., 1999a). DiI and lacZ exclusively labeled the ependymal layer and over time an increasing number of DiI-labeled cells were observed. Soon after labeled cells were found in the RMS and within 10 days the first DiI and lacZ labeled cells were seen in the olfactory bulb. DiI labeled cells in the olfactory bulb were immunoreactive for the neuron-specific proteins βIII-tubulin and Map2 (Johansson et al., 1999a). Furthermore, in response to injury a 50 fold increase in BrdU labeled ependymal cells of the spinal cord were demonstrated. EM analysis also revealed mitosis in the region with mitotic spindles orientated parallel to the luminal surface. Moreover, study revealed this population also demonstrated a highly enriched immunoreactivity for Notch 1, in a crescent of the luminal membrane of ependymal cells. This study demonstrated that ependymal cells are a proliferative population and that they respond to injury by increasing their proliferation rate, and that the majority of ependymal cells undergo asymmetric cell division.

To further investigate the stem cell potential of the ependymal layer, isolated and cultured cells from the ependyma separately from the subependyma and tested for the presence of NSCs using the *in vitro* neurosphere assay (Reynolds

and Weiss, 1996; Chiasson et al., 1999). The different cell populations were identified by the ability to incorporate BrdU under an established paradigm (Morshead and van der Kooy, 1992; Doetsch et al., 1997). Reports indicate that the ependymal cells can proliferate *in vitro* to form sphere-like structures. However, they do not have the ability to form secondary spheres after dissociation nor to produce neurons, but can generate GFAP expressing ependymal cells. This study did find a subpopulation of subependymal cells that do possess the self-renewing and multipotential characteristics of NSCs (Chiasson et al., 1999). It was concluded, that the adult forebrain neural stem cell resides within the subependymal compartment.

Subventricular Zone Astrocytes are Neural Stem Cells

It was later demonstrated that SVZ astrocytes, and not ependymal cells were the putative NSCs (Doetsch et al., 1999). A study was accomplished by eliminating populations of dividing cells and a sequential labeling paradigm. In order to eliminate the rapidly dividing cells, the anti-mitotic drug cytosine-b-D-arabinofuranoside (AraC) was infused into the surface of the brain of adult mice. This completely eliminated type A and C cells in the SVZ leaving only the type B cells and ependymal cells. Within 2 to 4 days the regeneration of type C followed by type A cells ensues. To confirm the identification of the regenerating cells immunostaining for GFAP was performed followed by microscopic and electron microscope analysis. These results confirmed that the only type B cells were regenerative. To support this conclusion dividing cells were identified by

exposure to thymidine-H^3 and followed by a one hour chase. The labeled cells showed that 98.7% were Type B astrocytes while the remaining cells were microglia. No evidence of ependymal cell labeling was seen. To determine the order of appearance of the different dividing cells dividing cells were eliminated by anti-mitotic treatment. It was logically reasoned that after anti-mitotic treatment, secondary precursors would only appear after the division of the primary ones. Thus these animals were examined 36 hours after the thymidine-H^3 injection and it was discovered that both types B and C cells were then labeled. A 5.5 day chase after thymidine-H^3 injection revealed types B, C and A cells were labeled. Thus the sequence of proliferation was determined to be that type B cells give rise to type C cells which give rise to type A cells. Subsequent retroviral labeling confirmed these results. In mice not treated with AraC, SVZ astrocytes specifically infected with a retrovirus give rise to new neurons in the olfactory bulb. This group also demonstrated that SVZ astrocytes give rise to cells that grow into multipotent neurospheres *in vitro*. To examine the proliferative extent of ependymal cells, mice were administrated BrdU for 2 weeks.

Sequence of Stem Cell Proliferation

Examination of SVZ revealed that none of the ependymal cells were labeled

Figure 2

with BrdU. Thus this group concluded that astrocytes act as neural stem cells in both the normal and regenerating brain (Doetsch et al., 1999).

Alternating views of the putative NSC still exist. In an attempt to correctly identify the developmental origin of adult neural stem cells the creation of a lox-Cre-based technique to specifically and permanently label a restricted population of striatal radial glia in newborn mice and adults (Merkle et al., 2004). In conjunction with the labeling of S-phase neurons with BrdU, they reported that within the first few days after labeling, radial glial cells gave rise to neurons, oligodendrocytes, and astrocytes, including astrocytes in the SVZ. They also reported that at all ages examined the RMS contained labeled migratory neuroblasts. Labeling showed that new neurons continue to be produced in the adult by precursors ultimately derived from radial glia. Further study revealed that both radial glia in neonates and radial glia-derived cells in the adult lateral ventricular wall generated self-renewing, multipotent neurospheres. It was thus proposed that the radial glial cells serve as neural and glial progenitors through out the life of the organism (Merkle et al., 2004).

Subgranular Layer Astrocytes are Neural Stem Cells

Using an approach similar to the one used to identify the neural stem cells in the SVZ, astrocytes have been identified as the neural precursors for the adult hippocampus (Seri et al., 2001). Evaluation by light microscopy has identified the precursors as having the characteristics of astrocytes. Authentication by electron microscopy (EM) shows that the precursor cells contain multiple processes with intermediate filaments rich in GFAP. Results from 3 independent experiments

supported this. It was found that proliferating sub granular layer (SGL) astrocytes quickly lose their GFAP immunoreactivity and structurally transform into cells that possesses previously not described as characteristics for proliferating cells. Anti-mitotic treatment with AraC and/or procarbazol resulted in the elimination of these cells from the SGL (as well as type A and C cells), but neurogenesis returned. Suggesting that since new neurons are born at a time when thymidine-H^3 labeled astrocytes were observed, that these cells behave as primary precursors in the SGL. To verify this hypothesis they labeled astrocytes with RCAS avian leukosis virus under the GFAP promoter. This retrovirus integrates into DNA, thus the progeny of infected GFAP-positive astrocytes can then be followed without dilution (Holland and Varmus, 1998). This labeling showed that SGL astrocytes, specifically labeled with an avian retrovirus, gave rise to granule neurons in the dentate gyrus. This group further identified the small dark cells which were previously thought to correspond to the primary precursors, are derived from the dividing astrocytes. These cells may be a transient cell type in the generation of new granule neurons. This work concluded with the identification of another population of astrocytes, type D cells, in the adult mammalian brain with the potential to generate new neurons in the hippocampus (Seri et al., 2001). To further explore the possibility that astrocytes are stem cells, a transgenic mouse line that expresses green fluorescent protein (GFP) under the nestin promoter was used (Filippov et al., 2003). *In vitro* studies demonstrate, that in the CNS, the intermediate filament nestin is expressed only in progenitor cells (Lendahl et al., 1990). The goal of this study was to examine the *in vivo* glial characteristics of

nestin-GFP-expressing, putative stem or progenitor cells in the adult dentate gyrus. This promoter, and the use of the nestin gene enhancer region, restricts reporter gene expression to neural stem and progenitor cells (Zimmerman et al., 1994; Kawaguchi et al., 2001; Sawamoto et al., 2001). Under this paradigm, they reported that nestin-GFP expressing cells of the dentate gyrus have two distinct morphologies. The first, a type 1 cell, expressed GFAP (but not of S100β, another astrocytic marker) and had an elaborate tree of processes through the granule cell layer and into the molecular layer with the cell body generally located in the granule cell layer. EM evaluation of type 1 cells showed vascular end feet of nestin-positive cells, thus supporting the differentiation of the astrocytes. The second cell type, a type-2, had a small round or oval nucleus and soma with little cytoplasm but lacked long processes. Most of the stubby cytoplasmic extensions of these cells were oriented tangentially to the granule cell layer. However, not all Type-2 cells displayed this uniform appearance. These cells also displayed bipolar and even multipolar morphologies with longer processes. They also reported that these cells often resided in the outer third of the granule cell layer or at the border of the molecular layer (Filippov et al., 2003). Further characterization of these populations demonstrated that nestin-GFP-expressing cells can also be distinguished by their voltage-gated channel expression patterns. Electrophysiological examination of type 1 cells on acutely isolated hippocampal slices showed passive current characteristics of astrocytes. The electrophysiological response of type 2 cells revealed two subtypes. Type 2-a cells responded with delayed-rectifying potassium currents. Also Type 2-b

showed neuronal membrane properties with voltage-gated sodium currents, potentially representing signs of the earliest steps of neuronal differentiation (Filippov et al., 2003).

Continued evaluation included analysis of 2 hour BrdU incorporation. The results indicated that both cell types were undergoing cell division at time of injection. However, it was reported that only type-1 cells showed constant co-labeling for astrocytic marker GFAP. In contrast, both cell types demonstrated a lack of immunoreactivity for S100β, a second astrocytic marker (Filippov et al., 2003).

Further discussion included the linkage of type 1 cells to the previously identified “B cells” and Type 2 cells correlating to type D cells (Seri et al., 2001). This was based on the expression of GFAP and structural morphology, followed by electrophysiological analysis and ending with BrdU incorporation. Concluding that the GFAP-positive population of putative progenitor cells *in vivo* is most likely not identical to the other mature astrocytes of this brain area but forms a distinct group, among other criteria characterized by their lack of immunoreactivity with antibodies against S100β. However, a question posed was whether nestin expression is a reliable marker for all stem or progenitor cells *in vivo* indicating more experiments were underway to answer this question. This data indicates that nestin-expressing progenitor cells of the adult dentate gyrus share certain astrocytic features but state that this feature alone may not qualify them as glial cells or astrocytes. They may just constitute an independent cell type with these characteristics (Filippov et al., 2003).

Glial Cell Dedifferentiation and Neural Stem Cell Progenitors

Many studies have reported astrocyte-like cells are NSC. To elucidate the mechanism behind this function, mature astrocytes harvested from human fetal brain were used. These cells were subsequently induced to express neuronal markers and exhibited neuronal morphology after treatment with FGF-1 and a cocktail of protein kinase activators. Western blots and immunocytochemical analysis revealed an up-regulation in immunoreactivity to nestin, β111-tubulin, neuron-specific enolase (NSE), and a transient expression of neurofilament-M (NF-M) proteins as compared to the controls. Analysis of the mRNA profile of treated cells confirmed these results with a significant expression increase of nestin, NF-M, NSE and synaptophysin. Moreover, mRNA expression Hes1, a transcription factor is important for maintaining the self-renewing ability of progenitors and for repressing the commitment of multipotent progenitor cells to a neuronal fate (Ishibashi et al., 1995), significantly increased during maturation period. In contrast, treatment failed to induce any notable change in electrophysiological membrane properties as compared to controls. This group suggested that treatment with protein kinase activators and FGF-1 synergistically induced a cascade of phenotypic changes as seen by scanning electron, phase contrast, and immunofluorescent microscopy. This change included the dedifferentatition of astrocytes and the transformation of these cells into neural precursors. Data supports this by the transient induction of the HES1 gene, followed by differentiation into neurons which are supported by the mRNA and protein expression of neuronal proteins (Pillai et al., 2006).

Astrocytes as Adult Neural Stem Cells

These reports and others have pushed to the fore the process of dedifferentiation as a mechanism for the generation of NSCs by astrocytes. The ability of mature astrocytes to dedifferentiate was previously observed (Leavitt et al., 1999), and it was demonstrated that mature cortical astrocytes may retain the capacity to transform into transitional radial glia, supporting neuronal migration after transplantation in adult mice. Reports show in injured adult rat spinal cord, astrocytes may dedifferentiate and acquire the potential of neural stem cells (Lang et al., 2004). Recent evidence also shows that other glia may retain these characteristics. It has been shown that oligodendrocyte precursor cells can differentiate to assume NSC characteristics, but this occurs only through a “transdifferentiation” phase in which they display astrocytic features (Kondo and Raff, 2000).

This exciting evidence opens up new avenues for NSC exploration. Harvesting of ubiquitous astrocytes from the CNS and inducing dedifferentiation followed by neuronal transformation can lead to new treatment paradigms, preventative therapies and lends hope to the repair of the CNS.

Conclusion

Reactive astrocytes are the hallmark of several CNS pathologies including cerebral vascular accident and traumatic brain injury. This endogenous response causes phenotypical as well functional changes in these cells. Phenotypic changes include hypertrophy, increased expression if intermediate filaments, and increased production of extracellular membrane components. While functional changes include the ability to mediate inflammation, excitotoxicity, edema, modulate the blood brain barrier, and form the glial scar. Thus, the activation of astrocytes plays a significant role in the repair of the CNS in response to injury. These cells maintain the tissue integrity following CNS damage and are involved in the protection of the microenvironment, isolation of the injured area and repair of the CNS. They do however; generate components which inhibit axonal regeneration which causes the loss of CNS function. Understanding the mechanism and cell signaling motifs may help promote the benefits of reactive gliosis and reduce the inhibition of repair of the CNS.

Astrocytes are clearly neural stem cells. Studies solidly demonstrate the self-renewing multipotential these cells posses. The function of dedifferentiation of glia, and more importantly astrocytes lends itself to increased study and the exploration of other CNS cells under this paradigm. The data suggesting glial cells must “transdifferentiate through an astrocyte-like morphology is consequential and lends hope to new paradigms for the repair of the CNS. Another field to be explored, in light of this information, is the mechanisms of

cellular turnover in the CNS and how these replacement cells maintain or augment cognitive function.

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